Using differential confocal microscopy to detect the phase transition of the membrane of giant unilamellar liposomes

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ABSTRACT

Giant unilamellar liposomes (diameter > 10 μm) are important for cell-membrane research and controlled drug-delivery. Mechanical properties of unilamellar liposomes in different physiological conditions are crucial for their applications. For example, liquid-gel phase transition of the bilayer membrane under different temperatures determines the stability and activity of liposomes. Bending rigidity is the most closely related mechanical property to phase transition. Owing to the flexible nature of bilayer membranes, accurate measurements of the bending rigidity of membranes are difficult. Here we report an all-optical technique to directly measure the bending modulus of unilamellar liposomes. We use differential confocal microscopy, a far-field optical profilometry with 2-nm depth resolution, to monitor the thermal fluctuations and the deformation of unilamellar liposomes. From the amplitude changes of thermal fluctuations along with temperature we can directly determine the phase-transition temperature of the membrane structure. We then employ optical force to induce sub-micrometer deformation of the unilamellar liposomes. From the deformation we obtain their bending rigidity with simple calculation. We find the bending modulus decreases from 8–11 pico-erg to 0.5–0.9 pico-erg as the liposomes are heated across the phase-transition temperature. All the measurements are done without contacting the samples, and the shapes of liposomes remain the same after the experiments.

Keywords: differential confocal microscopy, unilamellar liposome, bending rigidity, phase transition

1. INTRODUCTION

Soft materials, such as colloids, lipid membranes, liquid crystals, polymers, and the micro- or nano-structures they form, are among the important bases of modern applied sciences and biomedical engineering. For example, unilamellar liposomes are not only important model systems for cell-membrane research but also useful in controlled drug-delivery by encapsulating therapeutic components and transporting them into cells.[1] The mechanical properties of soft materials are important for the engineers and researchers to produce, manipulate, and understand these microstructures. Owing to the "softness," however, the measurement of mechanical properties is usually accompanied by intrusion, even unrecoverable damage, especially for the bilayer lipid membrane (BLM) structures. Because the surfaces of BLM specimens consist of only two layers of lipid molecules, it is difficult to directly apply stress on them and measure the mechanical properties. The main technical challenges are: (1) The measurement should be non-intrusive to avoid artifacts. Previous studies using atomic force microscopy on cellular membranes has the advantage of high spatial resolution, but it was found that the contact of the probe can penetrate the membrane.[2] (2) The stress-induced deformation should be sufficiently small to keep the corresponding strain in a linear reversible region, so that the mechanical properties can be analyzed with linear mechanical models. For ordinary unilamellar liposomes, this means nanometer deformation, hence nanometer resolution is required in measurement. (3) The measurement speed should be fast enough to track dynamic mechanical response, e.g., the thermal fluctuations of membranes. To date, micropipette aspiration is the most common method used for the measurement of the mechanical properties of BLM's, liposomes, polymersomes, etc.[3–5] However, micropipette aspiration can only perform static measurement, and it deforms the sample so large that a complicated mathematical model is required to analyze the data. In addition, when working with membranes composed of lipid mixtures, the high curvature in the area sucked into the pipette can cause changes in lipid composition.[4]

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In this paper we report an all-optical approach to detect the phase transition of unilamellar liposomes and to measure their bending rigidity under different temperatures. The key technique used in these measurements is differential confocal microscopy, a far-field optical profilometric technique with 2-nm depth resolution and 0.3-μm lateral resolution.[6] Because the probe of differential confocal microscopy is a microscope objective lens of which the working distance is on the order of 1 mm, the soft sample surface can be kept from being damaged. The measurement speed of differential confocal microscopy can be as fast as the response of optical detectors, therefore we can easily track the thermal fluctuations of liposome membranes. The high depth resolution of differential confocal microscopy enables us to detect the small deformation caused by femtonewton optical force. Since the deformation is less than 5% the diameters of liposomes, the data can be analyzed with simple analytical geometry, and the sample can always return to its natural shape after the experiments. With these unique features, it was pointed out that differential confocal microscopy is very suitable in the study of mechanical properties of BLM structures.[7]

In Section 2 we describe the sample preparation and the experimental setup. We will also explain the working principle of differential confocal microscopy. We show the experimental data and discuss the methods to deduce the bending modulus in Section 3. The measurements on the same liposome at different temperatures clearly shows the phase transition behavior, and the measured bending modulus is consistent with that obtained with x-ray scattering.

2. MATERIALS AND METHODS

2.1 Preparation of liposomes

Giant unilamellar liposomes made of dipalmitoyl phosphatidylcholine (DPPC) were prepared with the following procedures:[8] DPPC and charged phosphatidylserine were mixed at 9:1 by weight in chloroform:methanol (2:1 by volume) to make a 10 mg/ml lipid solution. About 0.1 ml solution was dried to form a lipid film on a culture dish, which was blown with dry nitrogen for 24 hours to remove the solvent in the lipid film. The lipid film was then prehydrated at 45° C with water-saturated nitrogen for 45–60 minutes. Next we added an aqueous solution containing 0.1-M sucrose and 0.1-M KCl into the culture dish. This solution would then be enclosed in the liposomes, therefore we termed it as the "inner solution." The culture dish was then sealed under dry nitrogen and incubated at 37° C for 24 hours. During the incubation the lipid film gradually stripped off the bottom surface of the culture dish and formed a "white cloud" floating in the solution, which contained the liposomes. Liposomes made with the above procedures could be stable in the culture dish for 2–3 days.

We then moved a drop of the liposome "white cloud" into another culture dish containing a 0.1-M glucose and 0.1-M KCl aqueous solution. This dish was then placed on an inverted optical microscope. Because the density of this outer solution was less than that of the inner solution, the liposomes stayed at the dish bottom after we kept the dish rest for one hour. Figure 1 shows an image of one liposome viewed through the inverted microscope. Through the experiments the liposomes were kept in the outer solution.

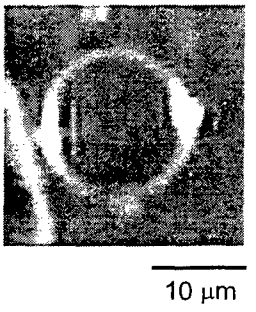
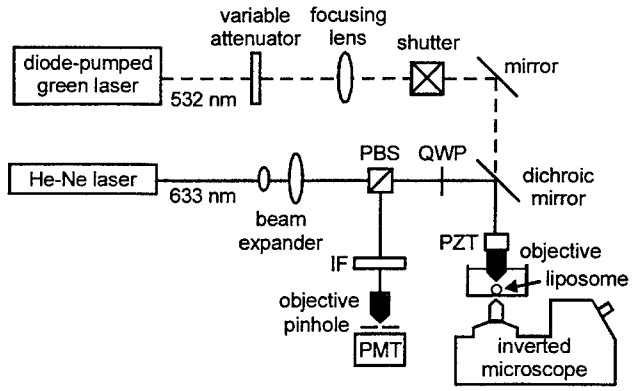
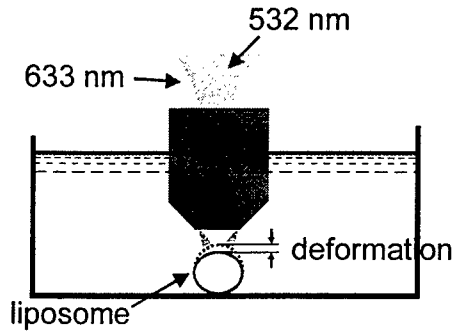


Fig. 1. Image of a DPPC liposome viewed by an inverted optical microscope. In order to enhance the contrast of the membrane, we raked the illuminating light source such that light can be reflected from the membrane into the objective lens of the microscope.



(a)



(b)

Fig. 2. (a) Experimental setup. IF, interference filter; PBS, polarization beam splitter; PMT, photo-multiplier tube; PZT, piezo-electric transducer; QWP, quarter wave plate. (b) The arrangement of beam sizes on the liposome. The 532-nm beam, used to exert optical force, was pre-focused such that its beam diameter was 14 μm on the liposome. The 633-nm beam was expanded to fill the whole input aperture of the objective lens, so that it probed the deformation only at the center of the 532-nm beam, where the deformation was uniform. The spot size of the 633-nm beam on the liposome was about 1 μm in diameter.

2.2 Optical setup

The experimental setup is shown in Fig. 2(a). Our set-up bore close resemblance to a conventional confocal microscope, where the probe light (the 633-nm beam), reflected from the liposome membrane and filtered by a 5-μm diameter pinhole at the conjugate focal point, formed the confocal signal.[9] For the operation of differential confocal microscopy, the focal plane was intentionally placed slightly away from the liposome surface, such that the position of the membrane was on a steep slope of the confocal axial response curve. This made the signal light that entered the confocal aperture extremely sensitive to the position of the membrane.[6] Owing to the low reflectivity of BLM's (10^{-3}–10^{-4}) in the surrounding solution,[10] the reflected signal light was measured with a photo-multiplier tube.

Two light sources of different wavelengths were used in the experiment. The 532-nm beam of 45 mW (measured after the water-immersion objective lens) was used to exert optical force on a single liposome, giving rise to a press force of 56 femtonewtons. The force f was calculated directly from the momentum conservation law: $f = 2Rnp/c$, where R is the reflectivity, n the index of refraction of the surrounding medium, p the optical power incident on the membrane, and c the speed of light in vacuum. The 633-nm beam of 70 μW was used as the probe beam to measure the deformation. These two beams were co-linearly focused by a 40× water-immersion objective lens with 0.75 numerical aperture (ICS Achroplan, Carl Zeiss, Oberkochen, Germany). The objective lens was mounted on a piezoelectric-transducer (PZT) driven objective

holder (PIFOC, Physik Instrumente, Waldbronn, Germany). Locations of the light spots on the liposome were monitored with an inverted optical microscope. The probe beam was expanded before entering the objective lens, such that it could be focused to a 1-μm diameter at the center of the 532-nm beam. To generate a uniform optical force, the 532-nm beam was pre-focused on the back focal plane of the objective lens, such that its spot size was as large as 14 μm in diameter on the focal plane (see Fig. 2(b)). The optical pressure produced by the 532-nm beam was practically constant in the beam center where measurements were made, and the beam-size variation was negligible within a few micrometers of distance along the optical axis. This arrangement ensured that the spatial distribution of the optical force was uniform.

2.3 Working principle of differential confocal microscopy

The axial response function of conventional confocal microscopy (see Fig. 3) is $I(z) = I(0)\sin^2(az)/(az)^2$, where I is the optical power of the signal, z the distance between the focal plane and the sample surface, and $a = 4\pi\sin^2(\alpha/2)/\lambda$ with $\sin(\alpha)$ the numerical aperture of the objective lens and λ the wavelength of the probe light.[9] Differential confocal microscopy takes advantage of the steep slope of the axial response function to obtain high depth resolution. The normalized slope of the axial response can be expressed as:

$$S(z) = \frac{1}{I(0)}\left|\frac{dI(z)}{dz}\right|. \tag{1}$$

In the linear slope region (shown as the black segments in Fig. 3) of the axial response curve, $S(z)$ is practically constant. Therefore the differential change of confocal signal is proportional to the displacement of the reflective surface. The proportional constants S and $I(0)$ are obtained before the measurement by scanning the focal plane through the sample surface with a high-accuracy transducer, such as the PZT-driven objective holder used in our experiment. In our setup $S \approx 1.0/\mu m$ in the linear slope region; hence a change of membrane position as small as 10 nm caused a readily detectable 1% change of the confocal signal. Such a sensitivity enabled us to measure liposome deformation down to a few nanometers.

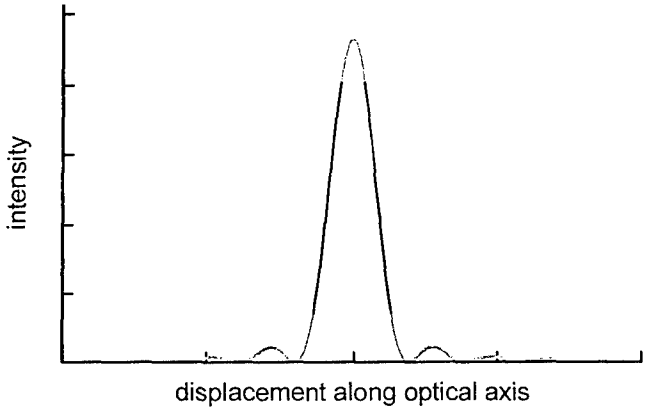


Fig. 3. Axial response curve of confocal microscopy. Black segments indicate the working region of differential confocal microscopy.

The depth resolution of our system was 2 nm, limited by the 0.2% power fluctuation of the power-stabilized He-Ne laser. As to the temporal resolution, since differential confocal microscopy relies on neither feedback control nor phase-locking mechanisms, the measurement can be as fast as the response of the photo-multiplier. However, in practice one has to set the detection time constant large enough in order to make the shot noise smaller than the power fluctuation. This is of importance for the conditions with weak optical signal.

2.4 Procedures of measurement

By observing the laser spots and the liposomes through the inverted microscope, we overlapped the laser beams and a single liposome laterally. Then we descended the objective lens along the optical axis and monitored the change of confocal signal of the 633 nm beam. When the confocal signal reached the first maximum after the objective lens was immersed into the outer solution, we were sure that the focal plane was right on the liposome surface. At this position we obtained $I(0)$. Then we raised the objective lens for a few hundreds of nanometers to place the liposome surface at the linear slope region of the confocal axial response curve. In the linear slope region we used a triangular high-voltage waveform to modulate the PZT objective holder and recorded the optical signal. From the displacement of the objective holder and the change of optical signal we determined the slope S. With $I(0)$ and S, from Eq. (1) we can determine Δz from ΔI. To match the requirements for high signal-to-noise ratio in different conditions of experiments, the signal amplification and the detection time constant were controlled by the biasing voltage of the photo-multiplier tube and a current amplifier. The amplified data were analyzed on-line with a fast Fourier transform spectrum analyzer, and stored in a personal computer through a 16-bit analog-to-digital converter.

We monitored the thermal fluctuations of the membrane before applying optical force to induce the deformation. In order to obtain sufficient signal-to-noise ratio and to cover the bandwidth of these fluctuations, here we set the measurement time constant to be 5 ms. It is well known that the thermal fluctuations of BLM reflect the mechanical properties. However, because of the low signal-to-noise ratio of previous measurements, complicated mathematical models were required to interpret the data.[11] Based on the high resolution of differential confocal microscopy we would directly observe the amplitudes of thermal fluctuations of BLM along with the temperature changes. Across the phase transition temperature the fluctuation behaviors had to be different, then we could determine the phase transition temperature of the membrane molecular structures.

Next we applied optical force on the liposome membrane by turning on the 532-nm beam, and measured the deformation of liposome along the optical axis (the z-axis). This experiment was to determine the bending modulus of the liposome membrane. To reduce the influence of thermal fluctuations, we fixed the power of 532-nm beam to be 45 mW and set the time constant of measurement to be 50 ms. For the calculation of bending modulus, the original diameter of the liposome was measured from the image obtained with the inverted microscope.

3. RESULTS AND DISCUSSIONS

3.1 Thermal fluctuations of membranes

The fluctuations of the shape of liposomes come from the Brownian motion of their membranes. From the models established in early studies on the flickering of erythrocytes, there exists a simple relation between the mean squared value of the change in diameters of such spherical vesicles and the temperature:

$$\left\langle |\Delta d|^2 \right\rangle \propto \frac{kT}{\kappa}, \tag{2}$$

where Δd is the change in diameter, k the Boltzmann's constant, T the absolute temperature, and κ the bending modulus of the membrane.[11] Therefore if we plot the squared values of fluctuation amplitudes as a function of temperature, we should obtain a straight line. When the temperature is changed across the phase-transition temperature, the slope of this straight line must be different according to the change of κ. This measurement can determine the phase transition temperature for further investigation.

Figure 4 shows the thermal fluctuations of a DPPC liposome measured with differential confocal microscopy. Because the liposomes were stable in the solution, we repeated the measurement on the same liposome at a temperature for four times, then changed the temperature. Each measurement lasted for 20 seconds. Since the amplitudes were very distributed even at a fixed temperature, we drew the histogram of the squared values of amplitudes in each measurement, and calculated their centroid as $|\Delta d|^2$:

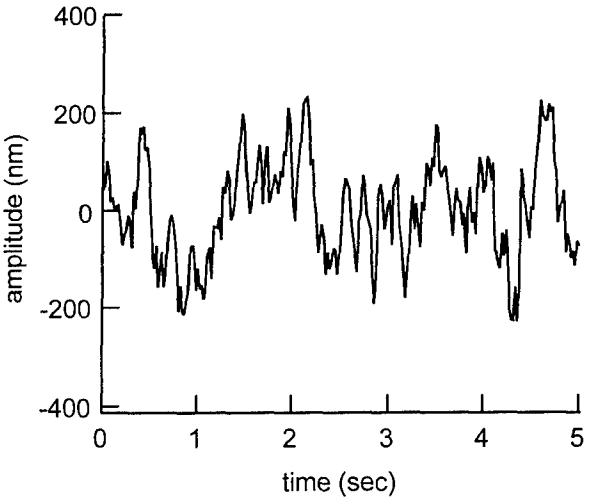


Fig. 4. Measured thermal fluctuations of a DPPC liposome at 45.5° C.

$$|\Delta d|^2 = \frac{\sum_i a_i^2 n_i}{\sum_i n_i}, \tag{3}$$

where a_i is the amplitude of the i-th interval of the histogram, and n_i is the number of amplitudes falling in the i-th interval. The amplitude range of each interval was set to be 20 nm. We calculated the average $|\Delta d|^2$ at each temperature, then plotted them as a function of temperature. The results are shown in Fig. 5. As we increased the temperature from 25.5° C to 51.5° C, we clearly observed the change of the slope of the fitting straight lines, caused by the change of bending modulus. In Fig. 5 the two lines intersects at 44° C, which approximates the phase-transition temperature ($T_c \approx 41°$ C) of DPPC in water.[1] The difference results from the difficulty to obtain reliable data near T_c. In experiments we found the reflectivity of DPPC membrane decreased dramatically as we increased the temperature to ~ 40° C. The reflectivity was so low that we could not record any data. Nevertheless, when we continued increasing the temperature to ~ 44° C, the reflectivity recovered to be

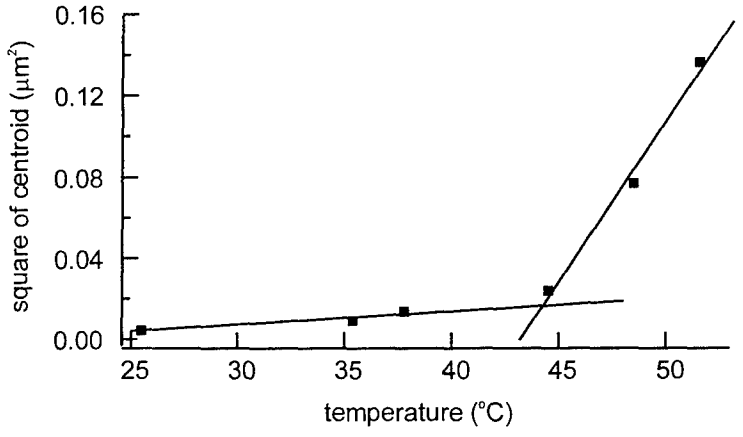


Fig. 5. Squared values of the centroid of measured amplitudes as a function of temperature. From the intersection of the two fitting straight lines we can approximately determine the phase-transition temperature.

about the same as in low temperatures. We found the same behavior of reflectivity as we decreased the temperature from 51.5° C to 25.5° C. This phenomenon indicates that the structure of molecules in BLM does not switch directly between the gel and the liquid phases. However, the discussions of the dynamical reconstruction of lipid molecules near the phase-transition temperature are beyond the scope of this study.

3.2 Bending rigidity of membranes

The second experiment was conducted to determine the bending rigidity of liposome membranes. We would first explain how we calculated the bending modulus from the diameter of a liposome and the sub-micrometer deformation caused by optical force.

Considering a liposome surface Ω, the free energy E can be expressed as follows:

$$E = \frac{\kappa}{2}\int_{\Omega}(c_1 + c_2)^2\, dA + \gamma \int_{\Omega} c_1 c_2 dA, \tag{4}$$

where c_1 and c_2 are the two principal curvatures, dA is the surface element, and γ is the Gaussian (or saddle) rigidity. For continuous perturbations of a closed surface, the term $\int_{\Omega} c_1 c_2 dA$ reduces to a constant.[11] In this case the changes in free energy can result only from the term of bending modulus κ. When the liposome is deformed by optical force, from energy conservation, the change in free energy is equal to the work W done by optical force. Since the optical force is known from the measured reflectivity, by measuring the changes in diameter of the liposome along the optical axis with differential confocal microscopy, we can determine W.

In order to obtain κ, the change in $\int_{\Omega}(c_1 + c_2)^2 dA$ has to be determined independently. Because there is no inner supporting structure inside a liposome, surface tension makes the liposome surface a perfect sphere. Thus without the optical force, $c_1 = c_2 = 1/r$, where r is the radius of the liposome, and $E = 8\pi\kappa + \gamma \int_{\Omega} c_1 c_2 dA$. When the liposome is deformed along the optical axis, its shape becomes an ellipsoid. Since the optical force is applied vertically, there is only one axis shorter than the other two. Therefore the principal curvature along the meridian can be calculated as

$$c_1 = -\frac{\left[2\left(\frac{dr}{d\theta}\right)^2 - r\frac{d^2 r}{d\theta^2}\right] + r^2}{\left[\left(\frac{dr}{d\theta}\right)^2 + r^2\right]^{3/2}}, \tag{5}$$

where θ is the azimuthal angle of the surface element from the short axis, and $r = \left[a^2 b^2 / \left(a^2 \sin^2\theta + b^2 \cos^2\theta\right)\right]^{1/2}$ with a the length of the short axis and b the length of the two long axes.[12] We applied the constant-surface-area constraint on the liposome surface Ω. Therefore, with the measured deformation along the optical axis we obtained both a and b. The other principal curvature is calculated by projecting the radius of curvature along the parallel onto the normal direction, *i.e.*, $c_2 = -r\cos(\alpha)/b^2$, where the cosine value of the angle α between the radius of curvature along the parallel and the normal direction is

$$\cos\alpha = \frac{2r}{\sqrt{4r^2 + \left(\frac{1}{r}\frac{dr^2}{d\theta}\right)^2}}. \tag{6}$$

Therefore the change in free energy is

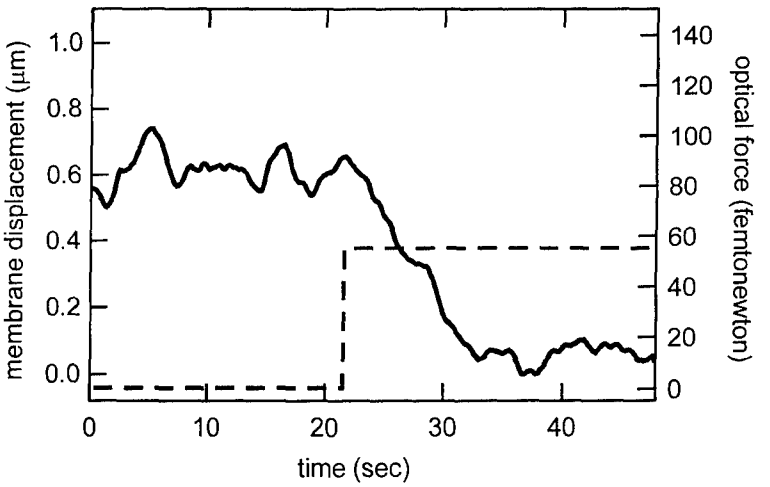


Fig. 6. DPPC liposome deformation along the optical axis under 56-femtonewton optical force at 27° C. Solid line, membrane displacement; dashed line, optical force.

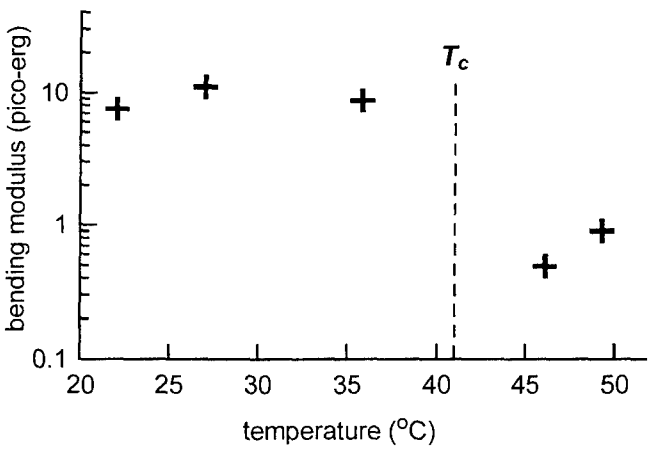


Fig. 7. Measured bending modulus of a DPPC liposome as a function of temperature. Across the phase-transition temperature T_c, the bending modulus decreases for an order of magnitude.

$$\Delta E = W = \frac{\kappa}{2}\int_{\Omega}\left(c_1 + c_2\right)^2 dA - 8\pi\kappa\,. \tag{7}$$

Figure 6 shows the liposome deformation under 56-femtonewton optical force at 27° C. The liposome was found to be deformed by ~ 600 nm. The corresponding work done by the optical force was thus 0.34 pico-erg. From the image obtained by the inverted optical microscope, the original diameter of this liposome was measured to be 14 ± 1 μm. With these data, from Eq. (7) we obtained κ = 11 pico-erg. Since this temperature was far below T_c, this κ value should be the bending modulus of the DPPC bilayer membrane in gel phase. With the same optical force, we found that the bending modulus remained almost the same for the temperature range of 22–36° C, as shown in Fig. 7. But when the temperature approached T_c, the low reflectivity again prevented us from taking reliable data. After the temperature was raised above T_c, we measured

κ values of 0.5–0.9 pico-erg. This was the bending modulus of DPPC membranes in liquid phase, which agreed with the recently published data obtained with x-ray scattering.[13] The sharp decrease of bending modulus in Fig. 7 clearly indicates the phase transition of the BLM structure. The results in Fig. 7 demonstrates both the correctness and the accuracy of our method to determine the bending rigidity of unilamellar liposomes in solution.

4. CONCLUSION

In this paper we describe an all-optical method to detect the structural phase transition and to determine the bending rigidity of unilamellar liposomes. We used differential confocal microscopy to monitor the thermal fluctuations of a DPPC liposome as the temperature changed, and observed the phase transition through the change of the linear relation between the squared amplitudes and the temperatures. Although an accurate measurement of the phase-transition temperature T_c was prevented by the low reflectivity of the BLM near T_c, we could obtain an approximate value with an error < 8% of other published values.

Using femtonewton optical force to deform the liposome, we measured the sub-micrometer deformation and calculated its bending modulus. Since the deformation was smaller than 5% of the diameter of liposome, only simple analytical geometry was required to calculate the bending modulus. The liposome returned to its original shape after each deformation, therefore we could repeat the measurement on the same sample for different temperatures. We found the bending moduli decreased for an order of magnitude when the temperature was increased across the phase-transition temperature. This macroscopic result clearly revealed the microscopic gel-to-liquid structural transition.

The technique presented in this paper is very convenient for biophysical experimentalists. The same measurement procedures are suitable to characterize kinds of molecular structures, such as BLM's, polymersomes, lipid tubules, etc. Thanks to the high resolution and long working distance of differential confocal microscopy, the samples can be measured *in situ*, and the data require only simple linear theoretical models to interpret. We believe differential confocal microscopy can serve as a daily tool for the studies related to membranes or other soft matters.

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